CARDIAC AND PULMONARY REPLACEMENT

CARDIAC GENE TRANSFER BY INTRACORONARY INFUSION OF ADENOVIRUS VECTOR–MEDIATED REPORTER GENE IN THE TRANSPLANTED MOUSE HEART

This study introduces a model for intracoronary gene transfer in murine cardiac isografts using adenovirus vectors. This approach may offer an opportunity to modulate alloreactivity after cardiac transplantation. Donor hearts were infected via the coronary arteries with a volume of 10^9 plaque-forming units per milliliter of a recombinant adenovirus containing the β-galactosidase–encoding gene (Ad.CMVLacZ). In a control group, 200 μl of normal saline solution was infused. The grafts were stored in 4° C cold saline solution for 15 minutes, then transplanted heterotopically into syngeneic hosts (B10.BR). The grafts were harvested at 3, 7, 15, or 30 days ($n = 5$ for each group) after transplantation, and β-galactosidase activity was assessed by histochemical staining (X-gal). All grafts were functioning when harvested. X-gal staining pattern was nonuniform with positive staining appearing in epicardial, myocardial, and endocardial cells, as well as in the vessel walls. The cells permissive to infection consisted predominantly of myocardial cells. The mean total numbers of β-gal–positive staining cells per slice were 68.7 ± 27.3 in the 3-day group, 330.4 ± 53.8 in the 7-day group, 151.3 ± 48.0 in the 15-day group, and 39.9 ± 10.8 in the 30-day group, thus peaking in the 7-day group ($p < 0.05$). Control isografts ($n = 5$), retrieved at day 30, revealed no staining activity. In conclusion, our model demonstrates that intracoronary gene transfer to the transplanted murine cardiac grafts is feasible at the time of harvest. Adenovirus-mediated gene transfer produces widespread gene expression which, though perhaps transient, does not adversely affect myocardial structure or function. This technology may allow modification of graft immunogenicity in the future through the production of therapeutic proteins sufficient to modulate local immune responses. (J THORAC CARDIOVASC SURG 1996;111:246-52)

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The development of methods to transfer genes into the heart has opened a new era in cardiovascular therapeutics. Through the localized expression of gene products, gene transfer approaches can provide insight into cardiac cell biology and may allow treatment of cardiac diseases such as transplantation coronary artery disease or graft rejection. Several methods of transfecting genes have been studied. In vivo, direct myocardial gene transfer through a needle has been investigated in the rat,[1-5] rabbit, microswine,[6] and dog.[7] However, the limited spatial extent of transfection has restricted the clinical applicability of this technique to human heart diseases.[4] By way of overcoming these limitations, the transvascular approach has been investigated as a potential target for gene therapy because of the

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large surface area and widespread distribution. Two types of approaches have been used to transfer genes into endothelial cells in vivo. First, genes have initially been transferred into endothelial cells in vitro, followed by reintroduction of the transfected cells into the vessel wall.[8-10] Second, the genes have been transferred in vivo directly into the vessel wall.[11-13] However, the fact that endothelial cells replicate slowly in the resting state may become a limiting factor for the use of vectors that depend on cell proliferation to express the exogenous genes.[14] A unique feature of adenovirus vectors is that they appear to be efficient in transferring foreign genes even into slowly replicating endothelial cells.[10]

The application of gene transfer techniques to the coronary vessels of cardiac allografts may offer insight into the alteration of alloantigen expression in the grafts and ultimately may allow the treatment of transplantation coronary artery disease or allograft rejection. Early detectable expression of genes after intracoronary infusion of a reporter gene at the time of harvest has been reported.[13]

In the present study, we evaluated the potential utility of adenovirus-mediated cardiac gene transfer by direct infusion of a reporter gene into the coronary arteries of the transplanted mouse heart.

Material and methods

Gene and adenovirus vector. The replication-defective recombinant adenovirus (Ad.CMVLacZ) encoding the *Escherichia coli LacZ* gene, capable of producing the enzyme β-galactosidase, was used as a reporter gene. The gene was modified by the addition of sequences encoding for a nuclear translocation signal and was placed under a control of the cytomegalovirus long terminal repeat. A volume of 200 μl of 10^9 PFU/ml* of viral particles was infused into the donor coronary arteries.

Animals. Fifty adult mice (7 to 10 weeks of age) of the B10.BR strain, weighing 17 to 22 gm, were obtained from Jackson Laboratories (Bar Harbor, Maine). They were housed under conventional conditions and fed a standard diet (Rodent Laboratory Chow, Ralston Purina Company, St. Louis, Mo.) and water. After completion of each procedure, the mice were allowed to recover with oxygen and local heat and transferred to their cages 24 hours after the operation with free access to food.

Heterotopic heart transplantation and intracoronary gene transfer. Cardiac isografts from B10.BR mice were transplanted into a second set of B10.BR mice by means of standard microsurgical techniques. After adequate anesthesia with 4% chloral hydrate (0.1 ml/20 gm of body weight, intraperitoneal injection) and methoxyflurane (inhalation), a sternal lid was lifted upward and fixated. Both the right and the left superior venae cavae were ligated. The donor heart was arrested by infusion of 0.5 ml of cold heparinized saline solution into the inferior vena cava (100 U heparin per milliliter saline solution). The left pulmonary artery was transected to vent the coronary sinus, and the ascending aorta was ligated just proximal to the origin of the innominate artery. The viral particles were then infused into the proximal aorta with a 27-gauge needle. In all cases, outflow of infused solution through the proximal cut end of the left pulmonary artery confirmed adequacy of delivery. The aorta, main pulmonary artery, and the three systemic veins were then transected distal to ligatures, followed by the en bloc ligature and transection of the pulmonary veins. The donor heart was preserved in 4° C normal saline solution during recipient preparation.

Through a midline abdominal incision, the recipient's infrarenal abdominal aorta and inferior vena cava were isolated and ligated both proximally and distally with 5-0 silk. After a longitudinal aortic incision, an end-to-side anastomosis between the donor ascending aorta and the recipient abdominal aorta was performed, followed by an end-to-side anastomosis between the donor pulmonary artery and recipient inferior vena cava. Both anastomoses were sutured with 10-0 nylon. The recipient's proximal aortic ligature was released first so that the graft would be initially reperfused with blood from the proximal aorta.

Expression of β-galactosidase. Histochemical staining was done so that β-galactosidase activity could be examined. Cross sections, 3 to 4 mm in height, were taken from the graft at midventricular level and snap-frozen in liquid nitrogen. Then 10 μm thick slices were cut at 200 μm intervals, followed by fixation with 1.25% glutaraldehyde. After being washed three times at room temperature with phosphate-buffered solution, the slices were stained for β-galactosidase with X-gal (5-bromo-4-chloro-indolyl β-D-galactopyronoside) for 4 to 6 hours, as previously described.[15] Six slices were examined for each mouse. For quantitative analysis of gene expression, the total number of cells positively staining for β-galactosidase was counted per slice under magnification (×40), and a mean value was calculated for six slices. Then, overall mean values were determined for each group (i.e., 3-, 7-, 15-, 30-day, and control groups). With the aforementioned regimen, *LacZ* expression was represented by a nuclear-dominant blue color.

Animal care. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Statistical analysis. Numbers are expressed as mean ± standard deviation of the number observed. The difference of the amount of gene expression was evaluated with analysis of variance. The level of significance was accepted as $p < 0.05$.

Result

Survival. All donor hearts resumed sinus rhythm after several minutes of reperfusion. Total ischemic

*PFU/ml = Plaque-forming unit per milliliter.

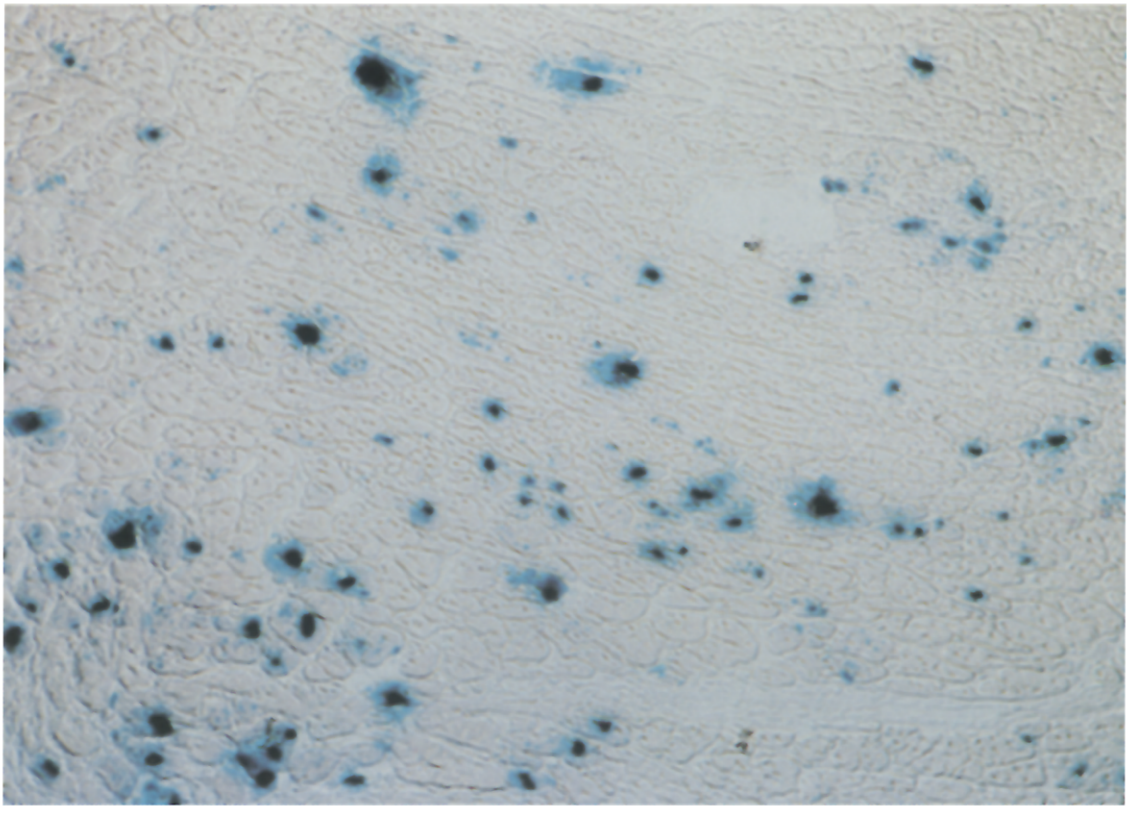

Fig. 1A. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in myocardial cells of coronary vessels.

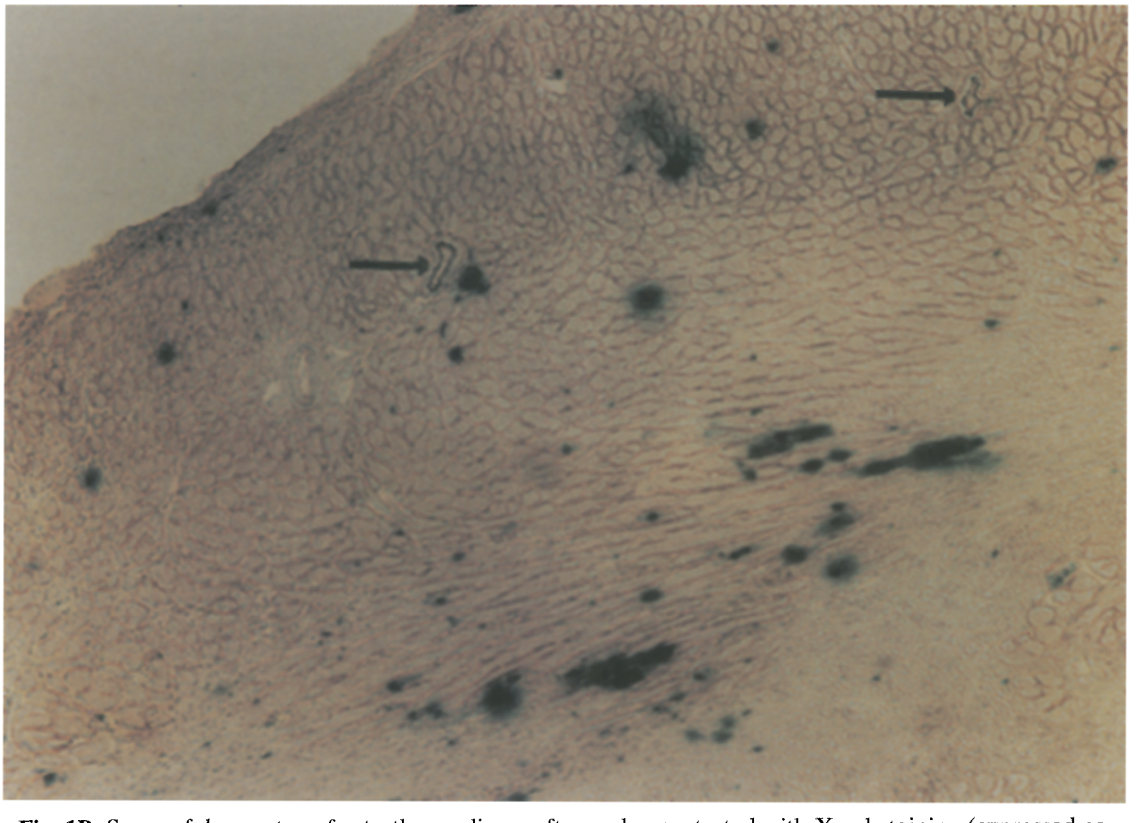

Fig. 1B. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in endothelial cells of coronary vessels.

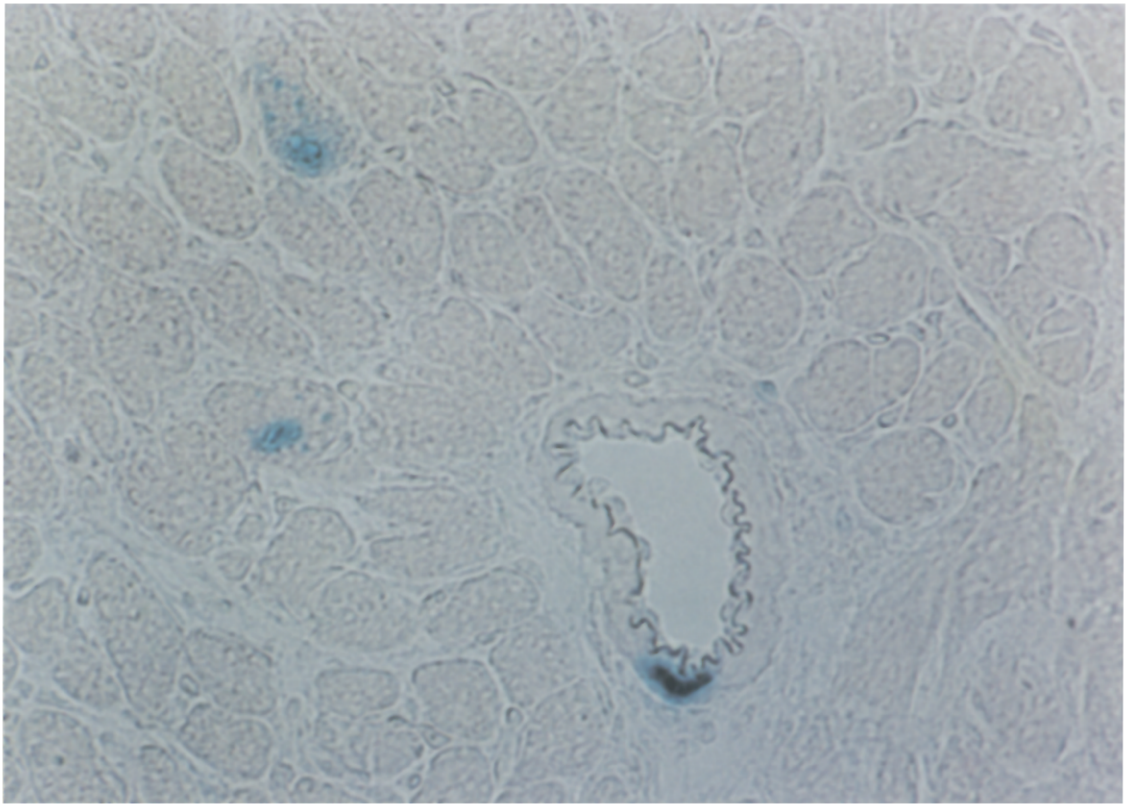

Fig. 1C. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in smooth muscle cells of coronary vessels.

time of the donor hearts ranged between 45 and 65 minutes. Nonsurvival rate, defined as survival for less than 24 hours after transplantation, was less than 10%. All animals surviving cardiac transplantation lived until they were sacrificed. All recipients had normal contractility of the transplanted heart when sacrificed. Aside from the β-galactosidase staining, the histologic appearances of both the experimental and control groups were normal.

Infection pattern and time course of expression. Successful gene transfer and expression of β-galactosidase was documented in all animals killed at 3 days. X-gal staining patterns revealed a nonuniform distribution, with blue-colored positively staining cells appearing in epicardial, myocardial, and endocardial cells, as well as in vascular walls (Figs. 1A, 1B, and 1C). The cells permissive to infection consisted predominantly of myocardial cells, and other myocardial structures were relatively well preserved (Fig. 1A). The mean total numbers of cells staining positively for β-galactoside per slice were 68.7 ± 27.3 in the 3-day group, 330.4 ± 53.8 in the 7-day group, 151.3 ± 48.0 in the 15-day group, and 39.9 ± 10.8 in the 30-day group, peaking in the 7-day group ($p < 0.05$). Control isografts ($n = 5$), retrieved at day 30, revealed no staining activity (Fig. 2).

Discussion

The present study proposes a model for adenovirus-mediated gene transfer to transplanted cardiac grafts and confirms the findings of recent studies on direct gene transfer into murine coronary arteries.[13] We observed expression of the *LacZ* gene, which is capable of producing the β-galactosidase enzyme, in the epicardial, myocardial, and endocardial cells, as well as in the coronary vascular walls, up to 30 days after transplantation, which demonstrates that this technique is a feasible means of transferring genes at the time of harvest.

Since Wolff and associates[15] demonstrated that murine skeletal muscle can take up and express genes that have been injected directly into the muscle, both skeletal and cardiac myocytes have been found to be efficient targets for gene transfer.[16] Several investigators have shown that expression of reporter genes injected into the skeletal or cardiac

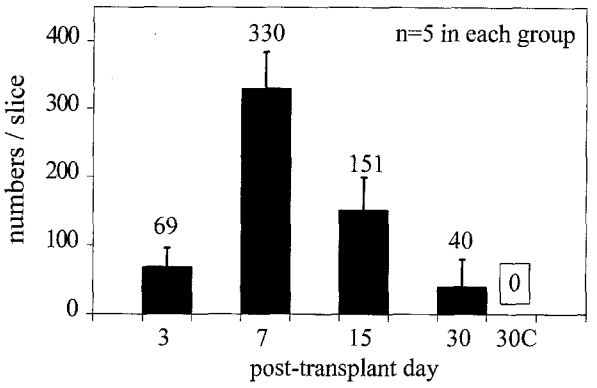

Fig. 2. Life span of transfected gene expression within the graft was analyzed by comparing the total number of cells staining positively for β-galactosidase per slice between the groups. Gene expression was detected in the 3-day group, peaking at 7 days, and gradually declined thereafter.

muscle was higher than for other injected tissues, including the brain, liver, spleen, uterus, stomach, lung, or kidney.[1, 2, 15] Although the reason for such apparently superior results remains unclear, it has been hypothesized that structural differences, including the presence of multinucleated cells, a sarcoplasmic reticulum, and a rich T-tubule system in skeletal and cardiac muscle, may allow better access of the gene to these cell populations.[1, 2, 15] The mechanism whereby the gene injectate is taken up by muscular tissues is unknown. It has been reported that only cells directly adjacent to the injection site are transfected, suggesting uptake of deoxyribonucleic acid (DNA) through leaking cell membranes.[1, 6] This finding, in addition to other experimental findings, raises questions about the clinical applicability of the direct injection method for transfection of human myocardium. These findings include the small number of myocytes that can be transfected in vivo (as few as 60 to 100 cells per injection), complete transfection only at the cells bordering the needle tract, and the occurrence of inflammation, myocardial necrosis, and scar formation along the needle tract.[1, 2]

Unlike direct myocardial injection, transfection via the walls of the coronary vasculature can be a useful means of gene transfer because of the potentially large absorptive area and widespread distribution. In vitro endothelial or vascular smooth muscle cells expressing a recombinant gene can be implanted on porcine endothelium-denuded vessel walls in vivo[8, 10] or on prosthetic interposition grafts of canine carotid arteries.[9] However, this technique requires prolonged occlusion of the target vessels to facilitate adenovirus attachment to the vascular wall,[8, 10] and it has a limited human application because of the practical difficulty of obtaining endothelial cells in advance.[11] Direct gene transfer into the unmodified vessels may overcome these problems. Previous reports have proved the feasibility of direct in vivo gene transfer into blood vessels.[11-13, 17] This study demonstrated that the recombinant adenovirus vector is able to transfer genes not only into the myocardial cells but also into the coronary arterial walls, the sites of local immune interactions after cardiac transplantation.

Recently, the adenovirus vector has been shown to be efficient in transferring exogenous genes into a variety of cells both in vitro and in vivo.[18-22] This vector has a number of advantages over other viral vectors. It is capable of infecting either nondividing or slowly proliferating cells, for example, vascular endothelial cells.[5, 23] Lemarchand and coworkers[10] have observed that replication-deficient adenovirus vector can mediate detectable gene expression in normal endothelial cells. The present study demonstrates that coronary vascular cells with β-galactosidase expression remain relatively intact microscopically. Other advantages of adenovirus vectors include the potential of generating viral stocks in excess of 1×10^{11} PFU/ml, an ability to accept heterologous genes up to 7.5 kb in length, no reported associations with human malignancies, and a history of safe administration in human vaccines.[24] Moreover, gene expression after adenovirus-mediated transfection in vivo could persist longer than other viruses. The mechanism underlying this phenomenon is unclear, but persistence of a moderate degree of viral replication may be responsible. A potential downside of this continued replication is that it may lead to a powerful host immune response to the infected cells.[23] A long-term model would be needed to examine this possible adverse effect.

An additional limitation regarding the generalized use of viral vectors has been the length of time required to generate each recombinant virus. Previous study has shown that an 18- to 24-hour incubation period is required to get the maximal lipofectin-mediated luciferase gene transfer in in vitro endothelial cell cultures.[11] Actually, such a long incubation period is not practical for in vivo gene transfer. In contrast, a considerable number (58%) of the endothelial cells expressed the β-galactosidase when the adenovirus vector (Ad.RSVβ-gal)

was in contact with the endothelial cells for even as short a period as 15 minutes in in vitro endothelial cell culture.[10] In the present study, the interval between the harvest and the reperfusion of the grafts (15 minutes of preservation in 4° C saline solution plus 45 minutes of room temperature exposure) was the incubation period. Clinically, the ischemic time of allografts provides a unique opportunity for access to the allografts and a window for direct gene transfer. Further studies are necessary, however, to demonstrate whether gene transfer can occur at hypothermic temperatures used for cardiac preservation.

The analysis of the time course of gene expression in our model corroborates the results obtained by others. Lemarchand and associates[10] observed that expression with adenovirus vectors peaked at 7 days and then declined, so that expression was no longer evident at 28 days in the in vivo carotid artery and umbilical vein of the lamb. Acsadi and coworkers[2] demonstrated that no β-galactosidase–positive cells were seen 25 days after direct myocardial injection of pRSVLacZ, suggesting that injected DNA remains episomal and is lost over time because of rapid target cell turnover. Additionally, they found that luciferase activity was stable for 60 days in cyclosporine-treated rats compared with unstable expression in untreated rats,[2] suggesting that an immune mechanism may be responsible for gene elimination. Nabel, Pautz, and Nabel[25] observed that β-galactosidase activity was expressed for at least 5 months after they transfected a segment of the iliofemoral arteries with retrovirus in vivo, and all three layers of the vessels including endothelial and vascular smooth muscle cells were infected by the retrovirus. Further efforts to increase the functional life span of viruses injected into infected organs need to be explored.

In summary, our model demonstrates that intracoronary gene transfer to the transplanted murine cardiac grafts is feasible at harvest. Adenovirus-mediated gene transfer produces widespread gene expression which, though perhaps transient, does not adversely affect myocardial structure or function. This technology may allow modification of graft immunogenicity in the future by using gene sequences capable of encoding immunosuppressive proteins. However, further efforts directed at increasing the level of expression and the functional life span of gene sequences need to be applied for this technique to be useful in patients.

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Discussion

Dr. John E. Mayer *(Boston, Mass.).* Genetic modification of donor allografts by means of viral vectors offers an exciting potential avenue by which both acute and chronic rejection may be modified.

Two questions have occurred to me. First, although much of your interest seems to have been focused on endothelial cells and transplant atherosclerosis, the great majority of infected cells were myocytes. Do you have any explanation for this? Does this finding imply that a different viral vector may be necessary or should the dose of the virus be greater?

Second, can you speculate on which genes should be transferred into the donor hearts? I have wondered whether the donor cells could be induced to produce the same HLA antigens as the recipient. Do you have any other ideas concerning which genes could be transferred into the donor organ?

Dr. Lee. Dr. Mayer, thank you for your comments and questions. First, our model was the first step study to evaluate the spacial and temporal expression pattern of adenovirus-mediated gene transfer in the transplanted mouse heart. It is true that the endothelial cells are thought to be among the useful targets to transfect genes to modulate local immune responses after cardiac transplantation because of their large absorptive area and widespread distribution. Recently, adenovirus vector has been shown to be an efficient tool to transfect genes even into nondividing or slowly dividing cells like myocardial or endothelial cells in both in vivo and in vitro studies. As you commented, our results revealed that myocardial cells were more efficiently transfected than the endothelial cells. However, the gene expressions in both myocardium and endothelium of the coronary vascular beds, the sites of local immune responses, were demonstrated in this study. This may justify the possible role of this approach to treat either transplantation coronary artery disease or graft rejection. The relative efficiency of transfection to the different cell types is to be studied further.

Second, there is no doubt that the long-term goal of these experiments is to develop the methods to express the transferred genes and to produce biologically active proteins in quantities sufficient to modulate local immune responses. Application of this technology using gene sequences for immunosuppressive cytokines including transforming growth factor-β (TGF-β) and interleukin-10 is currently underway with favorable results. Further efforts should be directed toward ways to increase the functional life span and rate of infection of these inserted gene sequences for this technology to be clinically applicable.

Dr. John H. Kennedy *(Cambridge, England).* Homocystine has recently been implicated as a risk factor in atherosclerosis, and the cytomegalovirus family of viruses is thought to invoke this response. Homocystine remains in tissue or body fluids for as long as 10 years. If you have any frozen material, it might be interesting to see whether a different virus would be more useful in your particular model if you are interested in graft rejection and accelerated atherosclerosis.